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Award Number: W81XWH-08-1-0321

TITLE: A Novel Anti-Beta2-Microglobulin Antibody Inhibition of Androgen Receptor Expression, Survival, and Progression in Prostate Cancer Cells

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REPORT DATE: May 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-05-2011	Annual	1 MAY 2010 - 30 APR 2011

4. TITLE AND SUBTITLE

A Novel Anti-Beta2-Microglobulin Antibody Inhibition of Androgen Receptor Expression, Survival, and Progression in Prostate Cancer Cells

5a. CONTRACT NUMBER

5b. GRANT NUMBER

W81XWH-08-1-0321

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Cedars-Sinai Medical Center
Los Angeles, CA 90048

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

β2-microglobulin (β2M) is a signaling and growth-promoting factor stimulating prostate cancer cell proliferation and progression. Blockade of the β2M signaling axis by anti-β2M monoclonal antibody (β2M mAb) resulted in the inhibition of androgen receptor (AR) and its target gene, prostate-specific antigen (PSA), and the induction of programmed death of prostate cancer cells in vitro and in vivo. We identified a new cis-acting element, sterol regulatory element-binding protein-1 (SREBP-1) binding site, within the 5'-flanking human AR promoter region and its binding transcription factor, SREBP-1, regulating AR transcription by β2M mAb in prostate cancer cells. Furthermore, we revealed a novel molecular mechanism by which SREBP-1 promotes prostate cancer growth, survival and progression. Alteration of SREBP-1 expression leads to regulate AR expression, cell growth, migration and invasion in prostate cancer cells. SREBP-1 also showed to induce fatty acid and lipid formation through increase of fatty acid synthase expression. Additionally, SREBP-1 induced oxidative stress and NADPH oxidase 5 (Nox5) expression in prostate cancer cells. In subcutaneous xenograft mouse models, SREBP-1 significantly increased LNCaP tumor growth and promoted prostate tumor castration-resistant progression. These findings provided a new concept to reveal the roles of β2M and SREBP-1, and their related signaling pathways, including AR, fat metabolism and oxidative stress, contribute to prostate cancer growth, survival and progression, and further provides a new potential target to prevent and treat prostate cancer malignancy by using β2M mAb and SREBP-1 blockers.

15. SUBJECT TERMS

anti-β2-microglobulin monoclonal antibody; androgen receptor; prostate cancer; sterol regulatory element-binding protein-1.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
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INTRODUCTION:

Prostate cancer progression from an androgen-dependent (AD) to an androgen-independent (AI) state is well recognized clinically as a fatal event. Androgen signaling mediated by the androgen receptor (AR), a ligand-activated transcription and survival factor, is known to play a key role regulating this lethal progression (1, 2). The central molecule of this project is β2-microglobulin (β2M). β2M is a non-glycosylated protein composed of 119 amino acid residues, and the mature (secreted) form contains 99 amino acid residues with a molecular mass of 11,800 Da (3, 4). β2M associates with the heavy chain of major histocompatibility complex class I (MHC I) on cell surfaces (5). This complex is essential for the presentation of protein antigens recognized by cytotoxic T lymphocytes (6) and serves as a major component of body's immune surveillance mechanism (7). We previously showed that β2M plays an unexpected role mediating prostate cancer osteomimicry, cell growth, survival and progression (8, 9), and AR expression. In this project, we evaluate the molecular mechanism of AR gene expression at the transcriptional level regulated by β2M during prostate cancer progression. We also focus on the β2M-mediated signaling and AR as a therapeutic target using a novel anti-β2M monoclonal antibody (β2M mAb) for the treatment of lethal prostate cancer malignancy. Recently, we identified a new *cis*-acting element, sterol regulatory element-binding protein-1 (SREBP-1) binding site, within the 5'-flanking human AR promoter region and its binding transcription factor, SREBP-1, regulating AR transcription by anti-β2M monoclonal antibody in prostate cancer cells (10). SREBP-1 is a key transcription factor for fatty acid and lipid biosynthesis (lipogenesis). Several reports have demonstrated that androgen biosynthesis and AR signaling in prostate cancer cells are intimately affected by lipogenesis (10-12). In this annual report, we will also provide evidence that SREBP-1 induces prostate cancer cell growth and promotes prostate tumor development and castration-resistant progression in animal models. Blockage of SREBP-1 activity by a small molecule inhibits cell proliferation and induces apoptosis in prostate cancer cells.

BODY:

1) Overexpression of SREBP-1 is associated with aggressive pathologic features in human prostate cancer. To study the clinical significance of a lipogenic transcription factor, SREBP-1, in prostate cancer progression, we determined the expression of SREBP-1 protein in human prostate carcinoma tissue microarray. We assayed SREBP-1 expression using a clinical prostate cancer progression set representative of tumors at different stages of the disease from normal/benign to localized cancer with different Gleason grades and scores (Fig. 1 and Table 1). SREBP-1 showed only 20% (3/15) positive expression in normal/benign prostate tissues, while expression of

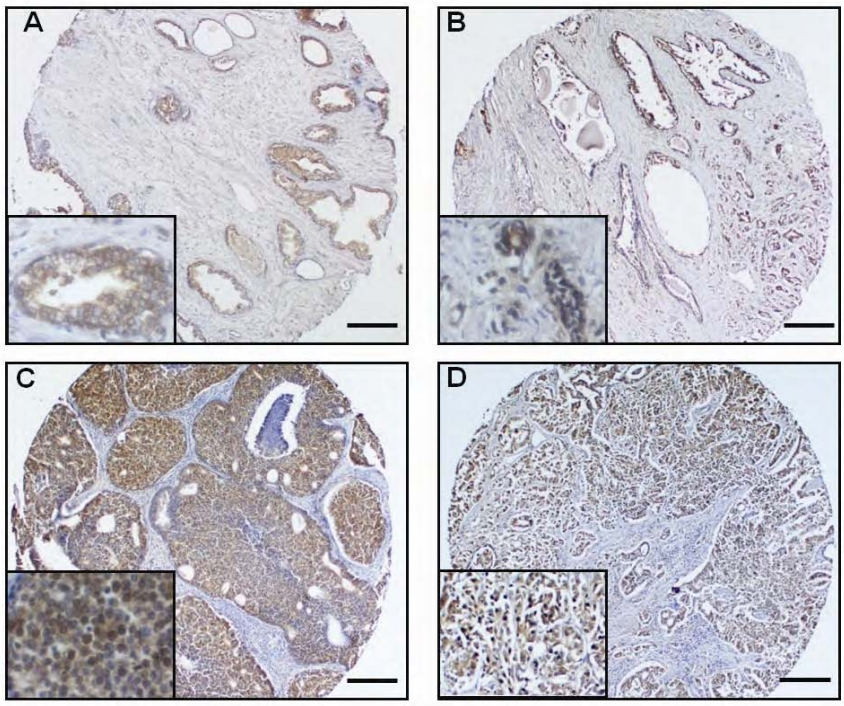

Fig. 1. Figure 1. Overexpression of SREBP-1 is associated with aggressive pathologic features in human prostate cancer. A human prostate carcinoma tissue microarray was used to determine expression of SREBP-1. A, Prostate normal/benign tissues; B, Pathology grade 3; C, grade 4; and D, grade 5 of prostate cancer tissues. SREBP-1 protein showed lower expression in human normal/benign tissues, an increasing expression with higher pathological grade. In particular, SREBP-1 was highly detected in nuclei in grade 4 and 5 prostate cancer. Scale bar = 275 μm.

SREBP-1 protein increased with higher pathological grades of disease [from 50% (grade 3) to 71% (grade 5); Table 1]. Interestingly, nuclear SREBP-1 was detected prevalently in grade 4 and 5 prostate cancers (Fig. 1C and D). Next, we compared expression of SREBP-1 in relation to high Gleason score (defined as ≥ 8). The results showed that SREBP-1 expression positively associated with increased Gleason score, from 31% positivity in Gleason score ≤ 7 to 70% positivity in Gleason score ≥ 8 (Table 1). Moreover, we noted that 78% (25/32) of SREBP-1 protein was found in the nuclei of Gleason score ≥ 8 tumors, but only 50% (4/8) in nuclei in Gleason score ≤ 7 tumors. Statistical analysis revealed that overall SREBP-1 expression levels were strongly correlated with pathological grades (P = 0.003) and Gleason scores (P = 0.003). These results suggested that expression of SREBP-1 protein is closely linked with the development of aggressive pathologic features in human prostate cancer. SREBP-1 may be a potential prognostic biomarker for human prostate cancer.

Table 1. Expression of SREBP-1 in human prostate carcinoma tissue microarray

Clinicopathological characteristics	The numbers of SREBP-1 expression, (%)	
	Positive	Negative
Pathology grade		
Normal/Benign (n#=15)	3 (20%)	12 (80%)
grade 3 (n=14)	7 (50%)	7 (50%)
grade 4 (n=27)	19 (70%)	8 (30%)
grade 5 (n=14)	10 (71%)	4 (29%)
Gleason score (GS)		
GS ≤ 7 (n=26)	8 (31%)	18 (69%)
GS ≥ 8 (n=46)	32 (70%)	14 (30%)

n: the numbers of samples

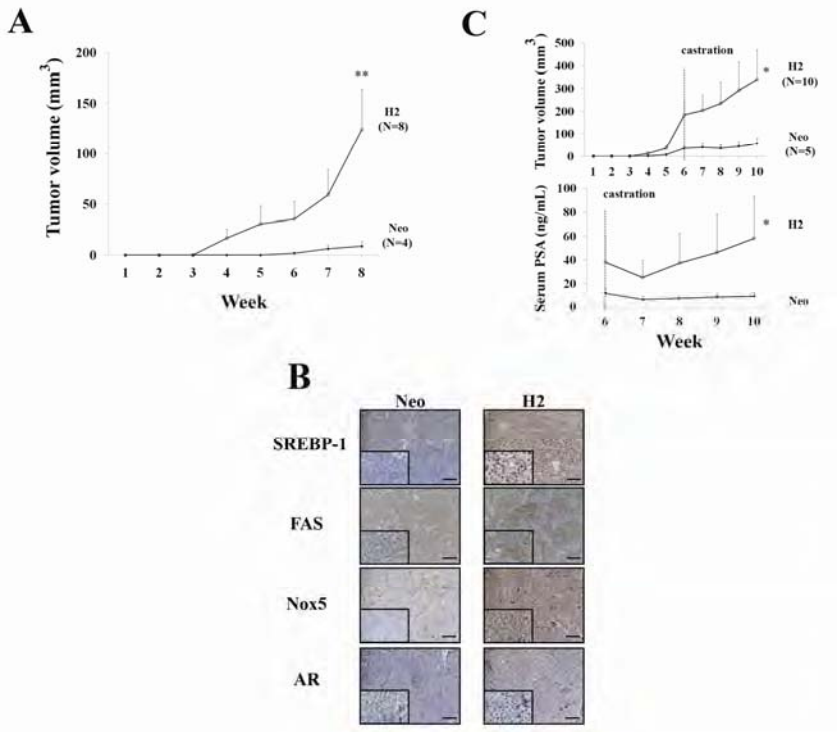

Fig. 2. SREBP-1 promotes human prostate tumor growth and castration resistance in mouse subcutaneous xenograft models. A, Tumor growth was assayed by tumor volume after inoculation of H2 and control Neo cells in mouse subcutaneous areas. SREBP-1 significantly induced the growth of H2 compared to Neo tumors. **, P < 0.005, significant differences from Neo tumors. B, IHC of subcutaneous Neo and H2 tumor specimens. H2 tumors highly expressed SREBP-1 (most in nuclei), FAS (cytoplasm), Nox5 (cell membranes) and AR (most in nuclei) proteins compared to Neo tumors. Scale bar = 100 μm. C, The mouse castration study. Tumor volumes of subcutaneous H2 tumors continuously increased after mouse castration (at week 6) compared to Neo tumors (the top panel). Serum PSA levels of both Neo and H2 tumor-bearing mice dropped at the first week post-castration (at week 7, the bottom panel). However, PSA levels of H2 mice significantly increased after four week castration (at week 10) compared to Neo group. *, P < 0.05, significant differences from Neo.

2) SREBP-1 promotes prostate tumor growth and castration-resistant progression in a subcutaneous xenograft mouse models. Because SREBP-1 expression increased in advanced form of human prostate cancer (14), we seek to determine if SREBP-1 confers growth advantages in hormone-naïve mice and resistance to tumor shrinkage in surgically castrated mice. We found SREBP-1 overexpressing H2 cells when inoculated subcutaneous developed 100% incidence of

tumor formation in mice; control Neo cells only exhibited 50% incidence of the tumor formation during an 8-week of observation. LNCaP classically showed less aggressive and low tumorigenic characteristics in mouse models (15). Furthermore, H2 tumors exhibited a 14-fold increased growth rate over that of the Neo tumors, as assessed by tumor volumes (Neo: 8.8±5.0 mm^3 and H2: 124.0±40.0 mm^3), after 8-week *in vivo* growth (Fig. 2A). Consistent with previous Western blot results, IHC data showed that H2 highly expressed SREBP-1 (most in nuclei), fatty acid synthase (FAS, a SREBP-1 targeted gene and has been shown to be a metabolic oncogene, cytoplasm), NADH oxidase 5 (Nox5, a SREBP-1 targeted gene and is a key enzyme for ROS generation; cell membranes) and AR (most in nuclei) in comparison to Neo tumors harvested from mouse subcutaneous space (Fig. 2B). Next, we sought to determine if SREBP-1 would be able to mediate castration resistance in prostate tumor xenografts grown in mice. Upon castration (at week 6), strikingly, subcutaneous H2 tumor growth continued compared to Neo tumors (Fig. 2C, the top panel). Serum PSA levels of both Neo and H2 tumor-bearing mice dropped at the first week post-castration (at week 7). However, serum PSA levels of H2 mice significantly rebounded after four weeks of castration (at week 10) compared to Neo mice (Fig. 2C, the bottom panel). These results suggested that SREBP-1 regulates prostate tumor occurrence, growth, and even resistance to castration in mice.

3) A new fat and weight reducing agent, 125B11, regulated gene expression and inhibited cell proliferation through blockade of SREBP-1 nuclear translocation in prostate cancer cells. A new small synthetic molecule, 125B11, has been reported to specifically inhibit SREBP-1 nuclear translocation and it downstream target gene expression, and further reduced fat and body weight in obese mice (16). First, we determined whether SREBP-1 nuclear translocation was affected by 125B11 in prostate cancer cells using immunofluorescence and Western blot analyses. Consistent with the previous data (16), 125B11 interrupted SREBP-1 nuclear translocation in Neo cells in immunofluorescence images (Fig. 3A). The results of Western blot also demonstrated that 125B11 inhibited nuclear translocation of mature SREBP-1 (68 kDa), and its target gene expression, including Nox5, FAS and AR (10) in both Neo and H2 cells (Fig. 3B). Next, we sought to examine if blocking SREBP-1 nuclear

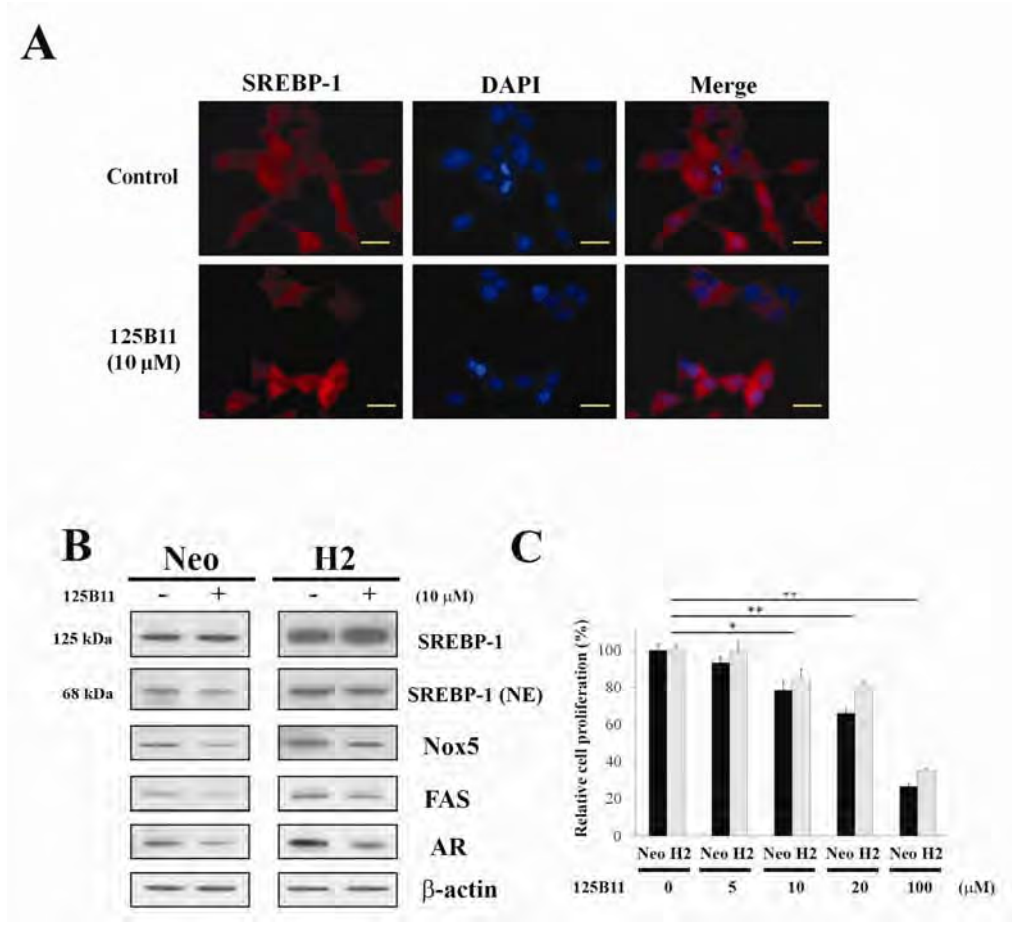

Fig. 3. A novel fat and weight reducing agent, 125B11, regulated gene expression and inhibited cell proliferation through blockade of SREBP-1 nuclear translocation in prostate cancer cells. A, 125B11 blocked SREBP-1 nuclear translocation in Neo cells assayed by immunofluorescence. Scale bars = 25 μm. **B**, 125B11 inhibited nuclear translocation of mature SREBP-1 (68 kDa), and expressions of Nox5, FAS and AR in both Neo and H2 cells. **C**, 125B11 affected cell proliferation (2-day) of Neo and H2 in a dose-dependent inhibition (0 to 100 μM). *, $P < 0.05$; and **, $P < 0.005$.

translocation by 125B11 affected cell proliferation in prostate cancer cells. As shown in Fig. 3C, 125B11 decreased cell proliferation of Neo and H2 cells with a dose-dependent pattern. These data suggest that by inhibition of SREBP-1 nuclear translocation, 125B11 decreased expressions of Nox5, FAS and AR, and reduced cell proliferation in prostate cancer cells.

4) 125B11 induced apoptotic death in prostate cancer cells. To investigate if blockade of SREBP-1 activity by 125B11 will induce apoptotic death in prostate cancer cells, we examined caspase activity and expression by enzymatic activity and Western blot analyses in Neo and H2 LNCaP cells. 125B11 significantly induced caspase3/7 activity in both Neo and H2 cells assayed by Caspase-Glo® 3/7 Assay Kit (Promega, Madison, WI, Fig. 4). The results of Western blot analysis of caspases showed that cleaved caspase-9, caspase-3, and PARP were increased by exposing Neo and H2 cells to 125B11 (Fig. 5). Collectively (Fig. 3-5) indicate that through interrupting SREBP-1 activity by a new agent, 125B11, decreased expressions of SREBP-1 downstream target genes, *Nox5*, *FAS* and *AR*, reduced cell proliferation and induce apoptotic death in prostate cancer cells.

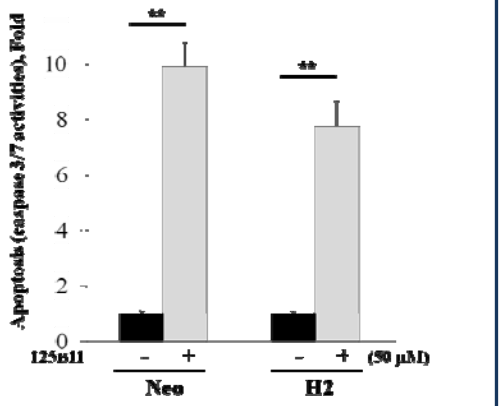

Fig. 4. 125B11 increased caspase3/7 activity in prostate cancer cells. 125B11 (50 μM, 48 h treatment) induced caspase3/7 activity in Neo and H2 LNCaP cells. **, $P < 0.005$.

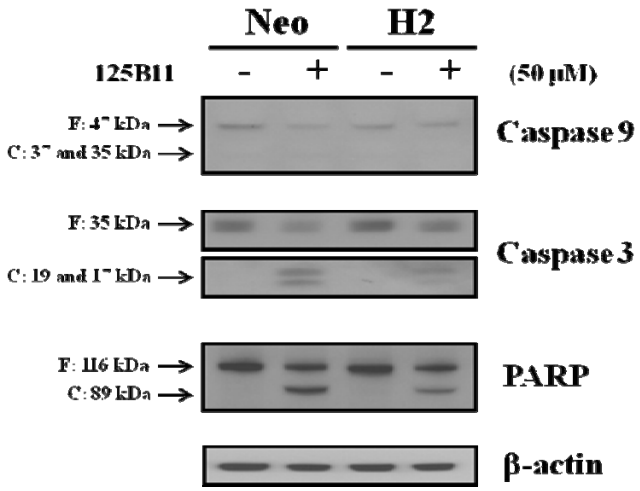

Fig. 5. 125B11 induced cell death of prostate cancer cells through an apoptotic cascade pathway. 125B11 (50 μM, 48 h treatment) activated the expression of cleaved caspase-9, caspase-3 and PARP proteins in Neo and H2 LNCaP cells as assayed by Western blot. F: full-length form; C: cleavage forms.

KEY RESEARCH ACCOMPLISHMENTS:

- SREBP-1 plays a key role in regulation of AR, FAS and Nox5 expression and cell viability in prostate cancer cells.
- In mouse xenograft models, we demonstrated that SREBP-1 promotes human prostate tumor initiation, growth and castration-resistant progression.
- Targeting SREBP-1 by a novel fat and body weight reducing agent, 125B11, interrupts SREBP-1 nuclear translocation and activity, and further inhibits cell growth and induces apoptosis in prostate cancer cells.

REPORTABLE OUTCOMES:

The third year of this DoD geant from May 1, 2010 to Apr 30, 2011: We collaborated with Dr. Leland Chung (Cedars-Sinai Medical Center) and published a peer-reviewed research article in *Cancer Research* (71: 2600-10, 2011; β2-Microglobulin induces epithelial to mesenchymal transition and confers cancer lethality and bone metastasis in human cancer cells). I presented a poster presentation in 2011 IMPaCT meeting (# PC073356-1798; poster title: A novel anti-β2-microglobulin antibody inhibition of androgen receptor expression, survival and progression in prostate cancer cells). Also, I received a Garber Cancer Research Award from Cedars-Sinai

Medical Center (project title: The SREBP-1/ROS signaling promotes prostate cancer development and progression). Currently, I am preparing a new manuscript regarding "the SREBP-1/AR/lipogenesis/oxidative stress study in prostate cancer" for this award.

CONCLUSION:

β2M is a signaling and growth-promoting factor inducing prostate cancer cell proliferation, survival and progression. Interrupting β2M and its related signaling pathways by a novel agent, β2M mAb resulted in the inhibition of AR and PSA expression and the induction of apoptosis of prostate cancer cells. The molecular mechanism of AR inhibitory expression by β2M mAb was through decreasing the interaction between a lipogenic transcription factor, SREBP-1, and its binding *cis*-acting element located in the 5'-flanking AR promoter region determined by electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation assay (ChIP). The functional study of SREBP-1 revealed that knocked-down or overexpressed SREBP-1 by utilizing a sequence-specific siRNA or an expression vector showed to decrease or increase total and nuclear AR protein in prostate cancer cells. SREBP-1 also induced *in vitro* cell proliferation, migration and invasion in prostate cancer cells. Additionally, SREBP-1 induced oxidative stress through increase of reactive oxygen species (ROS) levels and Nox5 expression in prostate cancer cells. In xenograft mouse models, strikingly, SREBP-1 increased LNCaP tumor initiation and growth, and promoted castration-resistant progression of huamn prostate tumor. A SREBP-1 activity blocker, 125B11, a weight and fat lowering agent, inhibits cell proliferation and induces apoptosis in prostate cancer cells. In summary, β2M mAb is a potent and attractive pleiotropic therapeutic agent to inhibit AR expression, cell proliferation, survival and fatty acid and lipid metabolism through down-regulation of a lipogenic transcription factor, SREBP-1, in prostate cancer cells. Targeting SREBP-1 by 125B11 also provides an alternative therapeutic approach for prostate cancer progression.

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APPENDICES: (Abstract for 2011 IMPaCT meeting**)**

A novel anti-β2-microglobulin antibody inhibition of androgen receptor expression, survival and progression in prostate cancer cells

PI: Wen-Chin Huang
Institute: Cedars-Sinai Medical Center, Los Angeles, CA

Background and objectives:
Prostate cancer progression is the underlying cause of mortality and morbidity of cancer patients. This lethal progression has been well documented to be associated with the androgen receptor (AR)-mediated signaling. To effectively manage prostate cancer, we must understand the operative factors and molecular mechanisms for this fatal disease. Recently, we identified a novel protein, β2-microglobulin (β2M), promoted prostate cancer cell growth and maintained prostate tumor survival and progression. Interrupting β2M by anti-β2M monoclonal antibody (β2M mAb) resulted in decrease of AR mRNA and protein expression, and induction of apoptotic death in prostate cancer cells. Based on these findings, we expect to develop a new and promising therapeutic approach using β2M mAb to treat lethal prostate tumor progression. The main objectives are: to investigate the molecular mechanisms by which β2M regulates AR expression in prostate cancer cells; and evaluate the anti-cancer efficacy of a novel β2M mAb.

Methodologies:
To identify the *cis*-acting element(s) in the 5'-flanking human AR promoter region, we conducted electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation assay (ChIP). Genetic manipulation techniques (overexpression and knock-down), cell proliferation assay, *in vitro* migration and invasion assays were performed to investigate the biological functions of a newly identified AR regulator, sterol regulatory element-binding protein-1 (SREBP-1), in prostate cancer cells. The immune-compromised mice bearing human prostate tumors were used to evaluate the potential toxicity and therapeutic efficacy of β2M mAb.

Results:
A new SREBP-1 binding site in AR promoter was identified to regulate AR transcription through β2M mAb action. SREBP-1 is a key transcription factor for fatty acid and lipid biosynthesis (lipogenesis). Overexpressing or knock-down SREBP-1 significantly affected lipogenesis, AR expression, and cell viability in prostate cancer cells. We further characterized the molecular mechanism by which β2M mAb interrupted survival signaling pathways in prostate cancer cells. β2M mAb decreased AR expression was through inhibition of MAPK and SREBP-1. By inactivation of MAPK, β2M mAb decreased prostate cancer cell proliferation and survival. By inhibition of SREBP-1, β2M mAb reduced fatty acid and lipid accumulation. These results provide for the first time a molecular link between the β2M intracellular signaling axis mediated by MAPK and SREBP-1 and involving lipid signaling, which collectively regulates AR expression and function.

Conclusions:
We revealed the pleiotropic β2M-mediated molecular mechanisms by which β2M mAb inhibited AR activity, lipogenesis and growth and survival in prostate cancer cells. Antagonizing β2M by β2M mAb may provide an effective therapeutic approach simultaneously targeting multiple downstream signaling pathways converging with MAPK, SREBP-1 and AR, for preventing prostate cancer cell growth, survival and progression.

Impact statement:
Prostate cancer progression is lethal and currently has no effective therapy. Our results demonstrated that blockade of the β2M signaling axis by novel β2M mAb resulted in inhibition of AR expression and lipogenesis, and induction of prostate cancer cell apoptotic death. Importantly, β2M mAb selectively killed prostate cancer

cells but not normal cells. Continued development of β2M mAb could result in a phase I clinical trial with the potential of controlling lethal prostate cancer malignancy in patients.

Cancer Research

β2-Microglobulin Induces Epithelial to Mesenchymal Transition and Confers Cancer Lethality and Bone Metastasis in Human Cancer Cells

Sajni Josson, Takeo Nomura, Jen-Tai Lin, et al.

Cancer Res 2011;71:2600-2610. Published OnlineFirst March 22, 2011.

Published OnlineFirst March 22, 2011; DOI:10.1158/0008-5472.CAN-10-3382

Cancer Research

β2-Microglobulin Induces Epithelial to Mesenchymal Transition and Confers Cancer Lethality and Bone Metastasis in Human Cancer Cells

Sajni Josson[1], Takeo Nomura[1,2], Jen-Tai Lin[1], Wen-Chin Huang[1], Daqing Wu[2], Haiyen E. Zhau[1], Majd Zayzafoon[4], M. Neale Weizmann[3,5], Murali Gururajan[1], and Leland W. K. Chung[1]

Abstract

Bone metastasis is one of the predominant causes of cancer lethality. This study demonstrates for the first time how β2-microglobulin (β2-M) supports lethal metastasis *in vivo* in human prostate, breast, lung, and renal cancer cells. β2-M mediates this process by activating epithelial to mesenchymal transition (EMT) to promote lethal bone and soft tissue metastases in host mice. β2-M interacts with its receptor, hemochromatosis (HFE) protein, to modulate iron responsive pathways in cancer cells. Inhibition of either β2-M or HFE results in reversion of EMT. These results demonstrate the role of β2-M in cancer metastasis and lethality. Thus, β2-M and its downstream signaling pathways are promising prognostic markers of cancer metastases and novel therapeutic targets for cancer therapy. *Cancer Res; 71(7); 2600–10. ©2011 AACR.*

Introduction

Bone is the second most common site of cancer metastasis, harboring over 70% of cancer metastases from prostate and breast cancers (1). Advanced-stage cancer patients develop bone metastases either with or without hormonal therapy, radiation therapy, chemotherapy, and immunotherapy, and currently there is no effective treatment. The pathogenesis of bone metastases remains poorly understood. So far there is no known transgene which reliably promotes cancer bone metastasis in immune-deficient mice or in immune-competent transgenic animals when expressed in cancer or normal cells. Here we demonstrated that overexpression of β2-microglobulin (β2-M) drives epithelial to mesenchymal transition (EMT) promoting lethal cancer bone and soft tissue metastases in human prostate, breast, lung, and renal cancers *in vivo*.

β2-M, a 11 kDa nonglycosylated protein, exists in all nucleated cells (2, 3). β2-M is involved in the regulation of the host immune response (4, 5). β2-M was reported by our laboratory (6–8) and others (9–11) as a growth factor and signaling molecule in cancer cells. β2-M expression increases during progression of human prostate cancer (9), breast cancer (12), renal cancer (13), lung cancer (14), colon cancer (15), and a number of liquid tumors (11). β2-M is a pleiotropic signaling molecule regulating protein kinase A, androgen receptor, vascular endothelial growth factor (VEGF; ref.7), fatty acid synthase (8), and lipid-raft-mediated growth and survival (11) signaling pathways. β2-M has multiple roles in cancer development and mediates tumorigenesis, angiogenesis, and osteomimicry (7). β2-M is also known to activate stromal cells such as mesenchymal stem cell (16), osteoblasts (17), and osteoclast (18). β2-M interacts can interact with major histocompatibility complex (MHC) class 1, classical, and nonclassical members. One of the nonclassical member is hemochromatosis (HFE) protein. β2-M knockout mice and HFE knockout mice have several identical pathophysiologic phenotypes, and develop symptoms of hemochromatosis involving iron overload and its associated diseases (19, 20). Several studies demonstrate the interaction between β2-M/HFE and its physical interaction with transferrin receptor, the primary mechanism for iron uptake in mammalian cells (21). In the present study, we demonstrated that HFE interacts with β2-M, modulating iron homeostasis, and governs EMT in cancer cells. We identified HFE as a β2-M receptor, which activates iron responsive HIF-1α (hypoxia inducible

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

S. Josson and T. Nomura contributed equally to the article.

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doi: 10.1158/0008-5472.CAN-10-3382

Published OnlineFirst March 22, 2011; DOI:10.1158/0008-5472.CAN-10-3382

factor-1α) signaling pathways and promotes cancer bone and soft tissue metastases.

Materials and Methods

Cell culture

Human androgen-refractory prostate cancer $ARCaP_E$ (androgen refractory prostate cancer—epithelial clone) and $ARCaP_M$ (androgen refractory prostate cancer—mesenchymal clone) and C4–2 prostate cancer [derived in the laboratory (22, 23)], MCF7 breast cancer and H358 nonsmall cell lung cancer cells (from ATCC) were cultured in T-medium (GibcoBRL) supplemented with 5% heat inactivated fetal bovine serum (FBS; Bio-Whittaker). Renal cancer SN12C cells (from ATCC) were cultured in minimum essential medium (MEM; GibcoBRL) with 10% FBS. Each had 50 IU/mL penicillin and 50 μg/mL streptomycin (GibcoBRL) in 5% CO_2 at 37°C. All cells were tested for mycoplasma (Mycoplasma detection kit (R&D systems), and were found to be negative.

Plasmid construction and stable transfection of β2−M expression vector

Mammalian expression plasmid for human β2-M in pcDNA3.1 was described previously (7). Empty pcDNA3.1 expression vector was used as control (Neo). MCF7, H358 and SN12C cells were transfected into plasmid with Lipofectamine 2000 (Invitrogen) and positive stable clones were established. Control and β2-M siRNA was retrovirally transfected into $ARCaP_M$ cells and are indicated as knockdown cells (KDI and KDII).

ELISA

β2-M protein concentration in blood and culture media was assayed by the Quantikine IVD human β2-M ELISA kit (R&D Systems).

Invasion and migration assays

Cancer cell invasion and migration were assayed in Companion 24-well plates (Becton Dickinson Labware) with 8 μm porosity polycarbonate filter membranes as described previously (24).

RNA preparation and reverse transcription (RT)-PCR analysis

Total RNA was isolated from confluent monolayers of cells using the RNeasy Mini Kit (QIAGEN). RT-PCR was performed as previously described (24).

Immunoblot analysis and flow cytometry

Western analysis was performed as previously described (24). The membranes were incubated with mouse monoclonal antibody against β2-M (Santa Cruz Biotechnology), E-cadherin (BD Biosciences), N-cadherin (BD Biosciences), Vimentin (Santa Cruz Biotechnology), HFE (Santa Cruz Biotechnology), and HIF-1α (Millipore) respectively, at 4°C overnight. Intracellular flow cytometric analysis was performed using BD CytoFix to permeabilize the cells followed by primary and secondary antibody treatments.

In vivo animal experiments

All animal experiments were approved and done in accordance with institutional guidelines. Four-week-old male or female athymic nude mice (19–21 g; BALB/c *nu*/*nu* mice, NCI) implanted with an 17β-estradiol pellet (NE-121, Innovative Research of America) subcutaneously were injected with 1×10^6 cells suspended in 10 μL sterile PBS into both tibias ($n = 8$). The estimated volume of bone tumors was calculated by 3 axes (*X*, *Y*, and *Z*) measured from a radiograph using the formula $\pi/6XYZ$ (25). Tumor size was also quantified by measuring hind limb diameter every 5 days. For intracardiac injection, anesthetized mice were injected with 5×10^5 cells/50 μL PBS/mouse into the left ventricle of the heart by nonsurgical means using a 28G1/2 needle (26). Metastases to distant organs were confirmed by radiography, necropsy, and histomorphology of the tumor specimens. At the time of sacrifice both hind limbs and tumor tissues were harvested for immunohistochemistry (IHC) and hemotoxylin and eosin (H&E) staining.

Immunohistochemistry

IHC was used to determine the level of protein expression in bone specimens. The following primary antibodies were used: E-cadherin (H-108; Santa Cruz Biotechnology) for E-cadherin, N-cadherin antibody (Abcam) for N-cadherin, Vimentin (V9; Santa Cruz Biotechnology) for vimentin, and β-2-Microglobulin (BBM.1; Santa Cruz Biotechnology) for β2-M. IHC staining were performed as previously described (24). Tartrate-resistant acid phosphatase (TRAP) staining was also performed to detect osteoclasts as previously described (24).

Immunoprecipitation

Immunoprecipitation was performed using the immunoprecipitation starter pack (GE Healthcare).

Lentiviral transduction

Lentiviral transduction was performed as per instructions (Sigma). Cells were selected using puromycin (4 μg/mL). Control cells which did not receive the viral particles died in 3 to 5 days. HFE shRNA transduced cells were characterized for HFE levels 7 to 10 days after transduction.

Iron measurements

Iron concentration was determined using induced coupled plasma mass spectroscopy (ICP-MS). Cells were grown to 10^7 cells and pelleted and digested using 3% nitric acid. Samples were diluted and analyzed by Perkin Elmer ICP-MS. The data are expressed as picomoles of metal.

Iron chelator and hypoxia treatments on $ARCaP_E$

$ARCaP_E$ cells were treated with 200 μmol/L of DES (desferal) for 48 hours. Then the DES was removed and replaced with normal media. A day later, cells were photographed and cell lysates were prepared for immunoblot analysis. $ARCaP_E$ cells were exposed to hypoxia (1% O_2, 5% CO_2, and remaining N_2) in humidified airtight chambers for 72 hours, cells were photographed and cell lysates were prepared for immunoblot.

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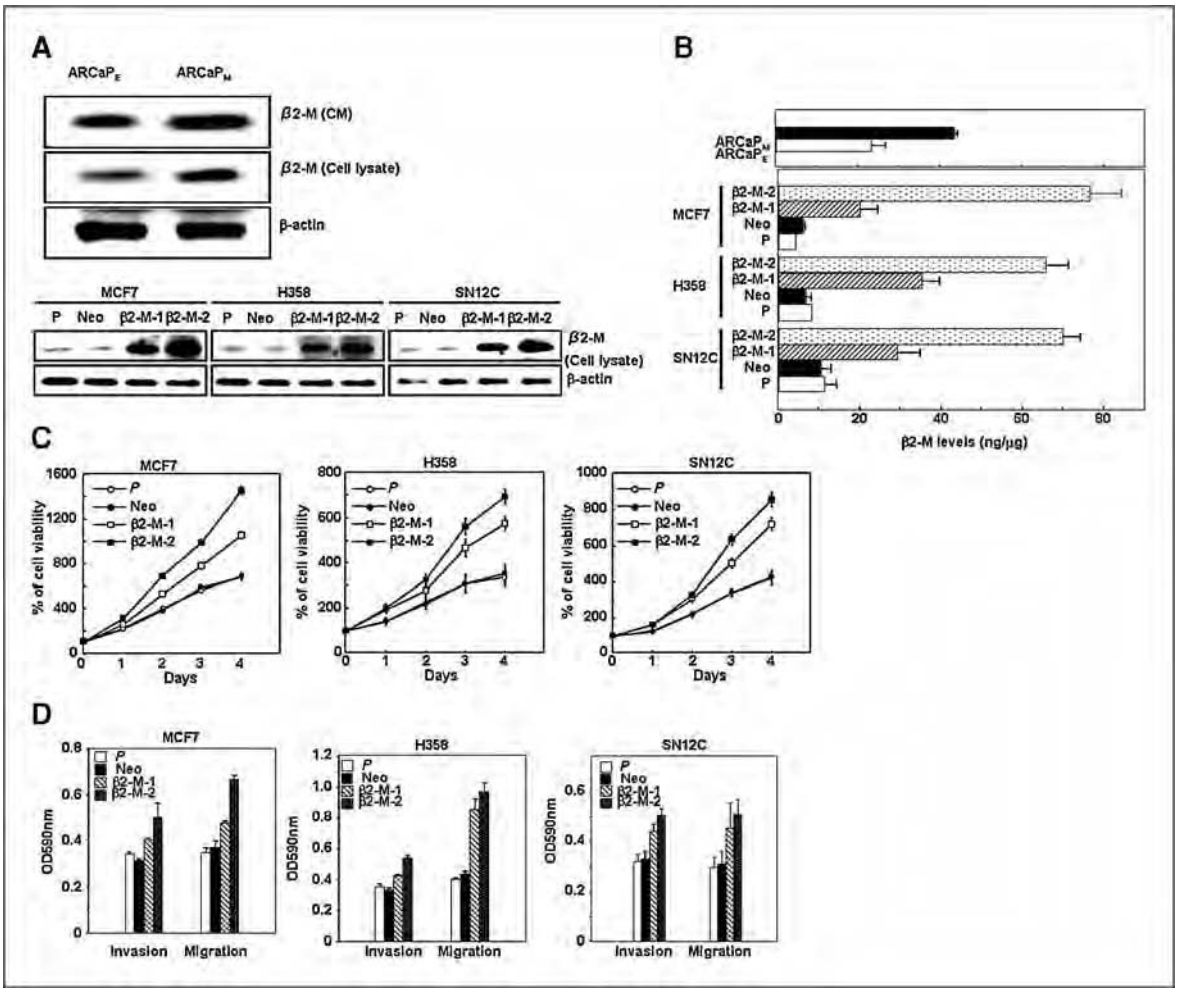

Figure 1. Characteristics of β2-M–overexpressing subclones in prostate (ARCaP), breast (MCF7), lung (H358), and renal (SN12C) cancer cell models. A, expression levels of β2-M protein in ARCaP$_E$ and ARCaP$_M$ in conditioned medium (CM) and cell lysates of MCF7 (breast), H358 (lung), and SN12C (renal) cancer subclones. B, β2-M concentration in conditioned media of ARCaP$_E$, ARCaP$_M$, MCF7, H358, and SN12C subclones, measured by ELISA. C, growth rates of Neo- and β2-M–overexpressing MCF7, H358, and SN12C cells. D, invasion and migration of Neo cells and β2-M–overexpressing MCF7, H358, and SN12C cells.

Statistical analysis

Values were expressed as means ± standard deviation. Statistical analysis was performed using Student's *t*-test or one-way ANOVA. Relationships between qualitative variables were determined using the χ^2 test. The estimated probability of survival was obtained using Kaplan–Meier methodology and differences were evaluated by log-rank test. Values of $P < 0.05$ were considered to be statistically significant.

Results

β2-M induces increased invasion and migration in breast, lung, and renal cancer cells

Our previous studies showed that ARCaP$_E$ cells, a subclone of ARCaP (androgen refractory prostate cancer) cells, underwent EMT, to become ARCaP$_M$ and gained increased growth and metastatic potential to bone and soft tissues (22). ARCaP$_M$ has 100% bone metastatic potential whereas ARCaP$_E$ has 12.5% (22). Accordingly, the steady-state levels of intrinsic β2-M protein were higher in ARCaP$_M$ than ARCaP$_E$ cells, as shown by western blot analysis in whole cell extracts and conditioned media (CM; Fig. 1A) and in CM by ELISA (Fig. 1B). To determine the function of β2-M we overexpressed β2-M in breast, lung, and renal human cancer cells. β2-M was overexpressed by a retroviral gene transduction method. A series of intermediate and high β2-M expressing human breast (MCF-7), lung (H358), and renal (SN12C) cancer cells were generated, characterized, and were confirmed by western blot analysis (Fig 1A) and ELISA of the CM (Fig. 1B). The high expressors of β2-M were designated MCF7/β2-M-2, H358/β2-M-2, and SN12C/β2-M-2, and the medium expressors of β2-M were designated MCF7/β2-M-1, H358/β2-M-1, and SN12C/β2-M-1 in each cell line. MCF7/P (parent), H358/P, and SN12C/P transfected with pcDNA3.1 vector alone

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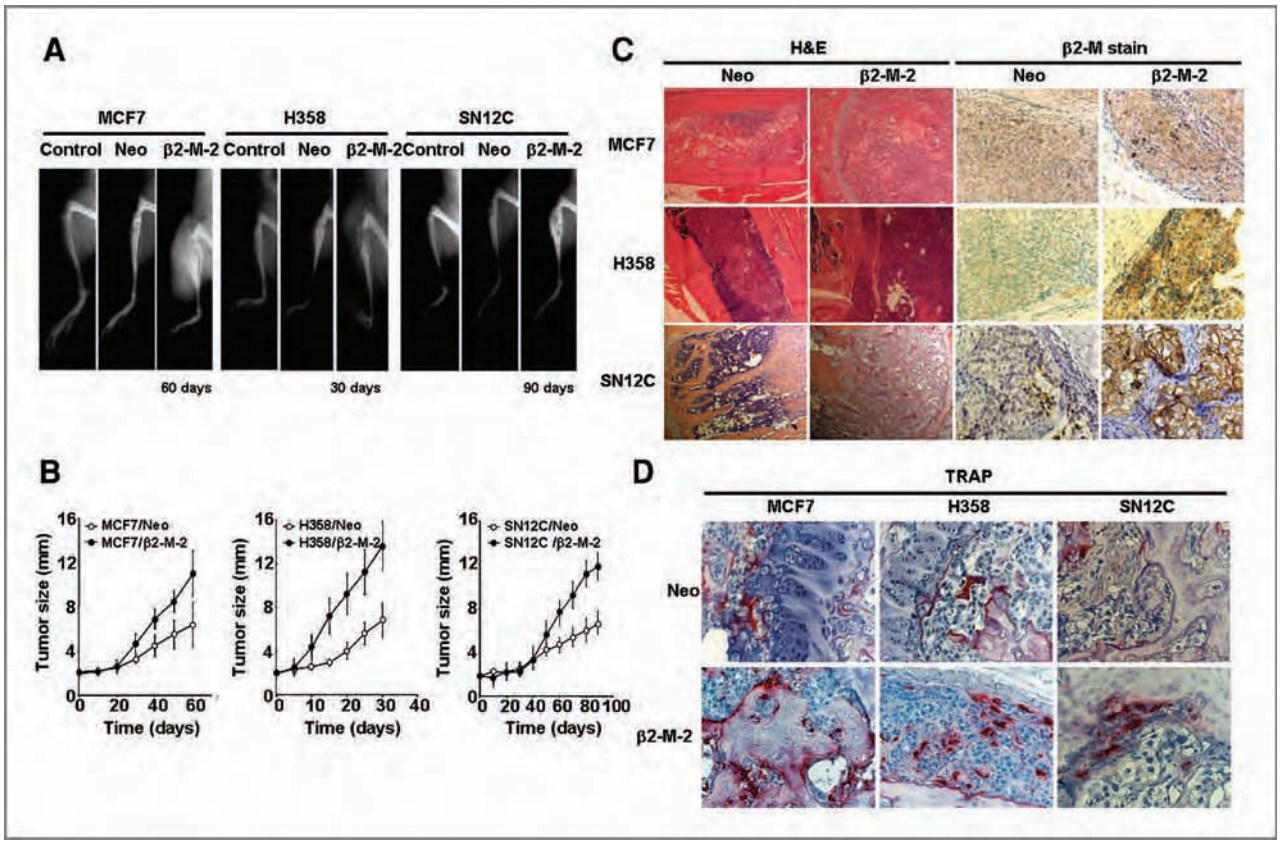

Figure 2. β2-M overexpression induces tumor growth of breast (MCF7), lung (H358), and renal (SN12C) cancer cells in mouse bone environment. A, Neo- and β2-M–overexpressing clones of MCF7, H358, and SN12C cells were injected intratibially into nude mice. X-ray images indicated that β2-M regulates the explosive growth of MCF7, H358, and SN12C tumor cells in mouse bone. B, tumor size was quantified every 5 days by measuring the hind limb X-ray images. Only 10-day tumor size was plotted. Each time point represents the mean ± SE of 8 tibias for each group. C, histomorphology (left) and immunohistochemical staining of β2-M (right) in mouse tibia injected with Neo- and β2-M–overexpressing clones of MCF7, H358, and SN12C cells. β2-M–overexpressing MCF7, H358, and SN12C tumors stained more intensely for β2-M compared to Neo controls. Magnification, H&E; 40×, IHC; 200×. D, TRAP staining of Neo- (top) and β2-M–overexpressing clones (bottom) of MCF7, H358, and SN12C cells from implanted tumor specimens. Magnification, 200×.

(MCF7/Neo, H358/Neo, and SN12C/Neo) served as controls. β2-M high expressors of breast, lung, and renal cancer had increased proliferation (Fig. 1C), migration, and invasion (Fig. 1D) compared to controls.

β2-M accelerated tumor growth of human breast, lung, and renal cancer with increased osteolysis in nude mice bone

Since $ARCaP_M$ cells were highly metastatic to bone, we compared the ability of Neo and β2-M–expressing MCF7 (breast), H358 (lung), and SN12C (renal) cancer cells to grow in the bone microenvironment in nude mice *in vivo*. β2-M–overexpressing clone (β2-M-2) and vector control clone (Neo) of MCF7, H358, SN12C were injected intratibially in the mouse skeleton, and tumor growth was assessed by radiography. Figure 2A shows that larger cancer cell-induced lesions with marked osteolytic responses and spotty foci of more intense osteoblastic lesions in mouse tibias implanted with β2-M compared to Neo-expressing cancer cell clones. Tumor volumes in β2-M-2-expressing clones were on average 3.5, 4.0, and 2.7 fold bigger than the Neo-expressing clones of MCF7, H358, and SN12C, respectively (Fig. 2B). Immunohistochemical analyses of the harvested tumors from mouse skeleton revealed increased β2-M staining in β2-M-2–expressing clones compared to Neo controls (Fig. 2C). Tartrate resistant acid phosphatase (TRAP) staining was performed to detect osteoclasts. The β2-M–expressing MCF7, H358, and SN12C cancer cells had a 3.6, 3.4, and 3.0 fold increases in osteoclasts compared to Neo controls (Fig. 2D). These results suggest that β2-M enhanced cancer cell mediated osteolysis by increasing the number of osteoclasts in breast, lung, and renal tumors grown in mouse skeleton.

β2-M expression positively correlated with the metastatic potential and lethality of human prostate, breast, lung, or renal cancer cells in immune-compromised mice

A comparative study was conducted using human prostate, breast, lung, and renal cancer cells expressing either basal or high levels of β2-M to assess cancer bone and soft tissue

Published OnlineFirst March 22, 2011; DOI:10.1158/0008-5472.CAN-10-3382

Table 1. Comparison of the metastatic potential of Neo and β2-M transfected cancer cells in athymic nude mice.

		Metastatic lesions					
Cell lines (number)	**Bone metastasis (%)**	**Lymphnode**	**Femur**	**Tibia**	**Lung**	**Adrenal**	**Others**
MCF7/Neo ($n = 14$)	7.1 (1/14)	0	1	1	1	3	1[a] (ovary)
MCF7/β2M-2 ($n = 14$)	42.9 (6/14)	1	6	5[b]	0	6	1[a] (ovary)
H358/Neo ($n = 16$)	6.3 (1/16)	2	1	0	2	1	
H358/β2M-2 ($n = 16$)	43.8 (7/16)	3[c]	7	6[b]	1	6[d]	1[a] (humerus) 1[a] (clavicle) 1[a] (paraspinal soft tissue) 1[a] (subcutaneous soft tissue)
SN12C/Neo ($n = 14$)	7.1 (1/14)	2	1	0	0	1	1 (kidney)
SN12C/β2M-2 ($n = 13$)	30.8 (4/13)	2	2	4[e]	1	1	1[a] (lower jaw)

[a]Same animal with femur and tibia metastasis.
[b]All of them had femur metastasis.
[c]Two of them had adrenal metastasis.
[d]Five of them had femur and tibia metastasis.
[e]Two of them had femur metastasis.

metastases and overall survival of the mice. Cells were injected intracardially into the left ventricles of nude mice. The presence of tumors in mouse skeleton and soft tissues was assessed by X-ray, physical palpation, and histopathology of tissue specimens harvested at the time of animal sacrifice. β2-M–overexpressed breast MCF7, lung H358, and renal SN12C cancer cells had significantly increased bone metastatic rates compared to controls (Table 1). β2-M–overexpressed breast MCF7, lung H358, and renal SN12C cancer cells had bone metastatic rate at 42.9% (6/14), 43.8% (7/16), and 30.8% (4/13), compared to mice inoculated with neo-expressing clones, which correspondingly were 7.1% (1/14), 6.3% (1/16), and 7.1% (1/14) (Table 1). Likewise, total soft tissue metastases to lymph nodes, liver, kidney, ovary, and adrenal glands were also moderately increased in β2-M–expressing cells of breast (MCF-7), lung (H358), and renal (SN12C) cells from 57.1%, 75%, 38.4% compared to neo-expressing controls, 35.7%, 31.2% and 21.4%, respectively (Table 1). β2-M expression was higher in metastatic bone tumors of $ARCaP_M$ and β2-M–expressing MCF7, H358, and SN12C tumors when compared $ARCaP_E$ or Neo-expressing control tumors by immunohistochemical analysis (Fig. 3A, right). Consistently, serum β2-M levels were also higher in mice injected with β2-M–overexpressing cells (Supplementary Fig. S1A). This level of β2-M is comparable to serum β2-M in human patients (27). Overall, $ARCaP_M$ and β2-M–overexpressing breast, lung, and renal tumors showed a more intense mixture of osteoblastic and osteolytic responses in bone compared with the specimens obtained from $ARCaP_E$ and Neo-expressing tumors (Supplementary Fig. S1B and C). The cumulative survival rate, as assessed by Kaplan–Meyer plots, of the mice injected intracardially with β2-M–expressing $ARCaP_M$, MCF7, H358, and SN12C cells also had significantly poorer prognosis compared to mice inoculated with Neo-expressing cells ($P = 0.0455$, $P < 0.0001$, $P = 0.0017$, and $P = 0.0075$, respectively; Fig. 3B). These results demonstrate that β2-M overexpression alone, in cancer cells, is sufficient to drive their subsequent skeletal and soft tissue metastases and caused lethality in experimental mouse models.

β2-M overexpression induced epithelial–mesenchymal transition in breast, lung, and renal cancer cells *in vitro* and *in vivo*

Both clinical and experimental data support the notion that cancer cells gain their metastatic potential by undergoing EMT (28). Using a robust ARCaP EMT model, we demonstrated a close association between EMT and prostate cancer bone metastasis (Fig. 4A). As a consequence of β2-M overexpression in breast, lung, and renal cancer cells, we observed notable EMT morphologic changes (Fig. 4A). β2-M–expressing $ARCaP_M$, MCF7, H358, and SN12C had decreased E-cadherin and increased N-cadherin and vimentin, compared to their neo-expressing controls at both the mRNA and protein levels (Fig. 4B and C). EMT markers were found to be stably expressed in harvested tumor tissue specimens as demonstrated by immunohistochemistry in β2-M–expressing MCF7, H358, and SN12C tumors when compared to the Neo-expressing control tumors (Fig. 4D). Similar results were observed in intratibial tumor tissue sections harvested from mice inoculated with the β2-M–expressing and neo-expressing cell clones (Supplementary Fig. S2). These results support the concept that EMT occurred subsequent to β2-M expression and this phenotype is stable *in vivo*.

Published OnlineFirst March 22, 2011; DOI:10.1158/0008-5472.CAN-10-3382

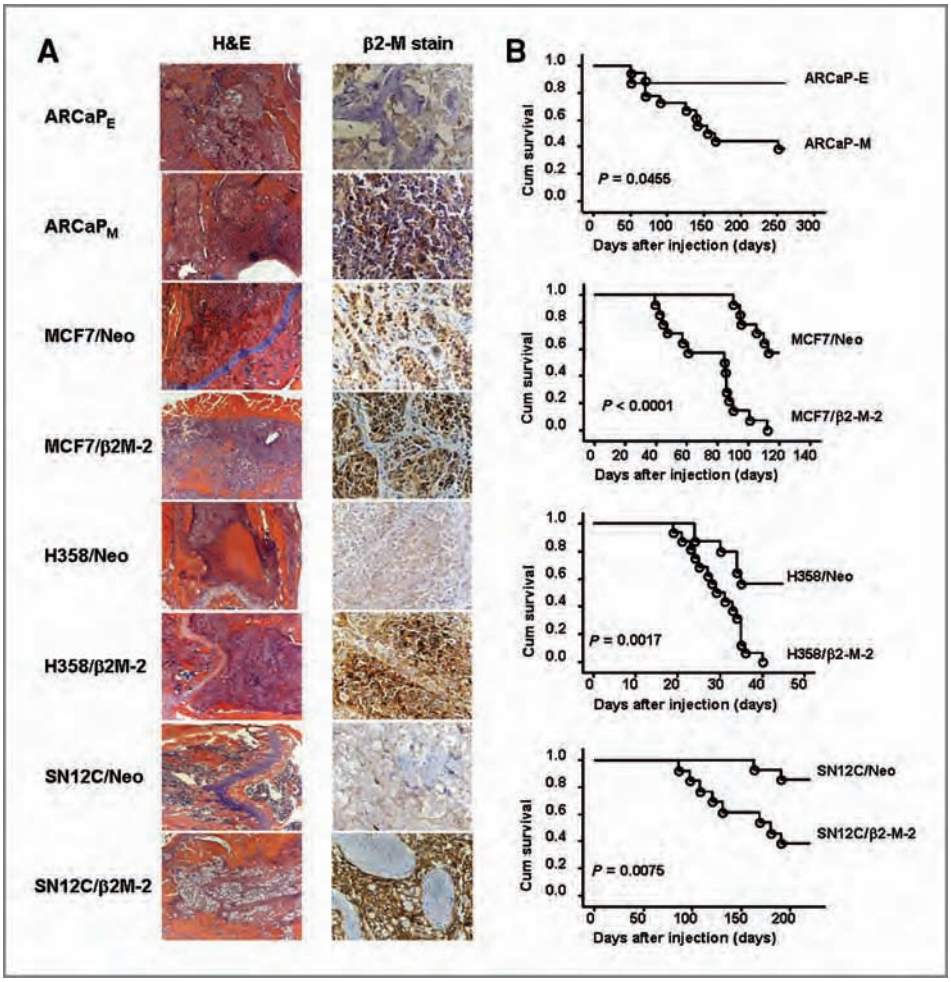

Figure 3. β2-M overexpression in prostate (ARCaP$_M$), breast (MCF7), lung (H358), and renal (SN12C) cancer cells confers increased lethal bone metastasis in nude mice. ARCaP subclones and Neo- and β2-M–overexpressing clones of MCF7, H358, and SN12C cells were inoculated intracardially into nude mice. A, histomorphology (left) and immunohistochemical staining of β2-M (right) in metastatic bone induced by intracardiac injections of ARCaP subclones and Neo and β2-M overexpressing clones of MCF7, H358, and SN12C in nude mice. Magnification, H&E; 40×, IHC; 200×. B, cumulative survival of nude mice inoculated intracardially with ARCaP$_E$ ($n = 8$) and ARCaP$_M$ ($n = 18$) and Neo- ($n = 14$–16) and β2-M–overexpressing clones of MCF7, H358, and SN12C ($n = 13$–16).

β2-M interacts with hemochromatosis (HFE) protein and inhibition of β2-M or HFE reverts EMT

To determine if inhibition of β2-M could reverse EMT [i.e., induce mesenchymal to epithelial transition (MET)], we performed studies knocking down intracellular β2-M with β2-M sequence-specific siRNA in ARCaP$_M$ prostate cancer cells. The control cells were treated similarly, using scrambled siRNA sequence (Scram). β2-M knockdown cells (KDI and KDII) had lower β2-M protein (Fig. 5A) and mRNA (Supplementary Fig. S3A) compared to ARCaP$_M$ Scram control. Both KDI and KDII underwent stable morphologic mesenchymal to epithelial transition (MET; Fig. 5B), which was accompanied by increased E-cadherin and decreased vimentin expression (Fig. 5A). Decreased β2-M also resulted in decreased invasion and migration (Supplementary Fig. S3B). HFE has been previously known to interact with β2-M. We tested in β2-M and HFE complex exists in prostate cancer cells. Physical interaction between β2-M and HFE as a complex was demonstrated by coimmunoprecipitation (co-IP) followed by western blot analyses (Fig. 5C). To determine the possible functional roles of β2-M/HFE complex-mediated EMT in ARCaP$_M$ cells, we knocked down HFE protein using HFE shRNA lentiviral constructs. Several stable clones were generated and KD$_{HFE1}$ and KD$_{HFE3}$ knockdown were used for further EMT characterization. KD$_{HFE1}$ and KD$_{HFE3}$ had significantly decreased HFE protein levels (Fig. 5D). Decreased HFE protein also resulted in decreased expression of vimentin and a moderately increased expression of E-cadherin (Fig. 5D, Supplementary Fig. S4D). Decrease in HFE also downregulated the expression levels of β2-M, thus reducing the β2-M/HFE complexes. Inhibition of HFE in C4-2 prostate cancer cells using a similar method resulted in decreased HFE and in increased E-cadherin (Supplementary Fig. S4A). We observed that downregulating HFE protein switched the morphology of ARCaP$_M$ cells to a cobblestone-like appearance, much like ARCaP$_E$ cells (Fig. 5D). Thus, disrupting the function of the β2-M/HFE complex by either HFE or β2-M knockdown is sufficient to reverse β2-M mediated EMT in prostate cancer cells. HFE-knockdown ARCaP$_M$ and C4-2 cells also had decreased invasive and migratory activity compared to control cells (Supplementary Fig. S4B and C).

Iron modulated EMT in cancer cells

β2-M protein is known to directly regulate iron levels in cells, in which β2-M/HFE complex block transferrin receptor 1 and prevent iron uptake. β2-M and HFE knockout mice have

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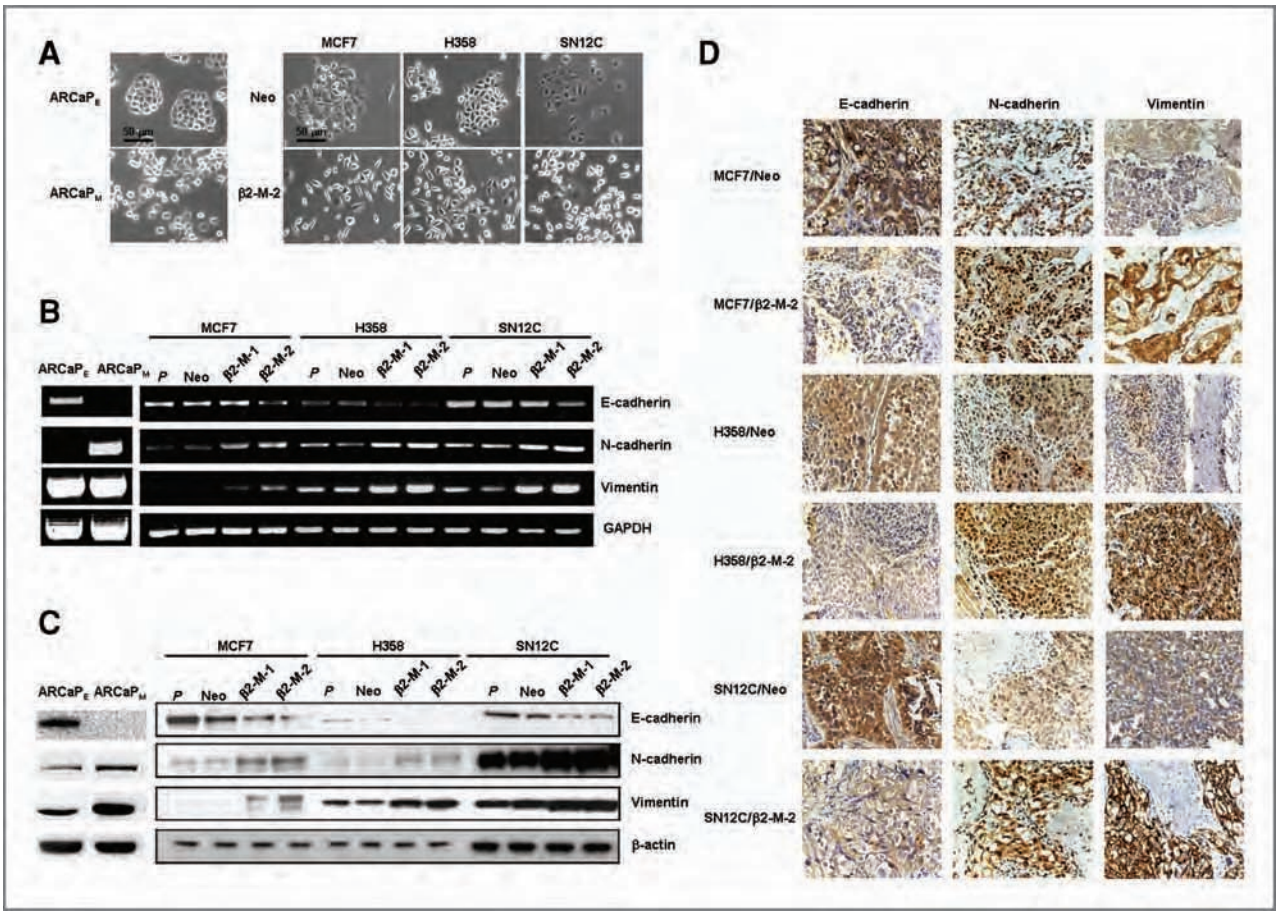

Figure 4. β2-M overexpression promotes EMT in breast (MCF7), lung (H358), and renal cancer cells (SN12C) *in vitro* and *in vivo*. A, ARCaP$_E$ had cobblestone morphology and ARCaP$_M$ had a spindle-shaped morphology. Neo cells of MCF7, H358, and SN12C cells had a cobblestone epithelial morphology whereas β2-M–overexpressing clones had a mesenchymal spindle-shaped appearance. B, EMT markers by RT-PCR of ARCaP$_E$ and ARCaP$_M$ and β2-M overexpressing MCF7, H358, and SN12C cells and controls. C, immunoblot analyses of ARCaP$_E$ and ARCaP$_M$ cells and β2-M overexpressing MCF7, H358, and SN12C cells and controls. D, expression of E-cadherin, N-cadherin, and vimentin in metastatic bone specimens from Neo- and β2-M–overexpressing clones of MCF7, H358, and SN12C.

iron overload (20). We hypothesized that β2-M overexpression in ARCaP$_M$ cells decreases iron and induces iron responsive HIF-1α (29). HIF-1α was previously shown to be elevated in mesenchymal ARCaP$_M$ cells compared to epithelial ARCaP$_E$ cells under normoxic conditions (30). We tested if cellular iron levels were lower in β2-M higher-expressing ARCaP$_M$ cells compared to β2-M lower-expressing ARCaP$_E$ cells and in HFE knockdown cells, using inductively coupled plasma mass spectroscopy (ICP-MS). Intracellular iron was significantly lower in ARCaP$_M$ compared to ARCaP$_E$ cells, KD$_{HFE1}$ and KD$_{HFE3}$ knockdown cells (Fig. 6A). To determine if iron could regulate EMT we used iron chelator to induce EMT like changes. Since the epithelial cancer cells (ARCaP$_E$, KD$_{HFE1}$, and KD$_{HFE3}$ knockdown cells) had slightly higher basal iron compared to ARCaP$_M$, we used iron chelator (desferal) on ARCaP$_E$ cells. Iron chelation increased HIF-1α and induced mesenchymal characteristics [Fig 6B(i) and (ii)]. We tested if HIF-1α can promote EMT in ARCaP$_E$ cells in response to hypoxic conditions. Hypoxia, upregulated HIF-1α, and ARCaP$_E$ cells exhibited mesenchymal like characteristics compared to cells maintained under normoxic conditions [Fig. 6C(i) and –(ii)]. β2-M knockdown cells had decreased HIF-1α measured by intracellular flow cytometry (Supplementary Fig. S3C). These results collectively demonstrate that β2-M expression in ARCaP$_M$ cells leads to decreased iron and increased HIF-1α, which induces EMT in prostate cancer cells.

In summary, β2-M can drive EMT, increase cancer bone and soft tissue metastasis and cause death in mice. β2-M mediates this process by interacting with a β2-M receptor, HFE, which together control intracellular iron homeostasis, activating HIF-1α, to promote EMT and increase lethal cancer cell metastases (Fig. 6D).

Discussion

The role of β2-M has long been documented in several solid and liquid cancers, but its mechanism of action is poorly understood. In this study, we documented for the first time that β2-M overexpression can drive EMT and promote the growth, invasion, and metastasis of human prostate, breast,

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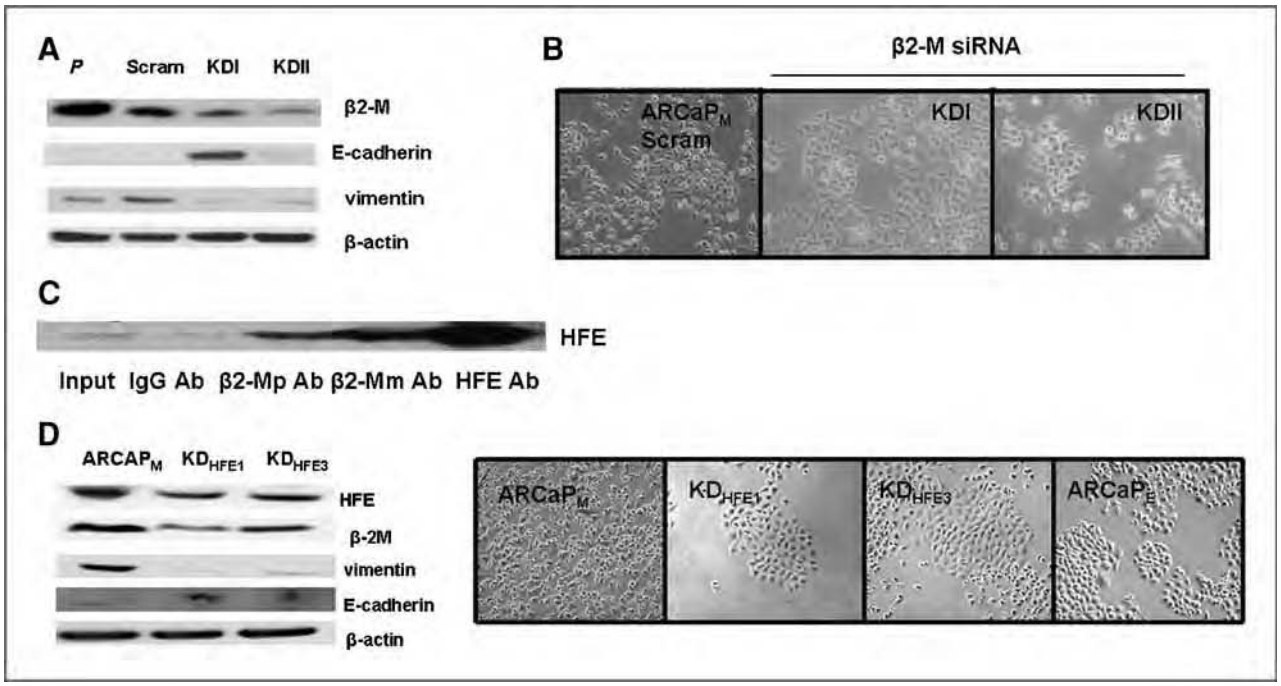

Figure 5. Inhibition of β2-M or HFE reverts EMT in prostate cancer cells. A, β2-M levels in ARCaP$_M$ control (Scram) and β2-M knockdown ARCaP$_M$ cells (KDI and KDII) by western blot analyses. Expression levels of β2-M, E-cadherin, and vimentin were analyzed. B, morphological changes in Scram and KDI and KDII cells. C, immunoprecipitation using polyclonal and monoclonal anti-β2-M Ab, and anti-HFE Ab. Western analysis of HFE. IgG Ab used as control. D, western analysis of HFE and EMT markers (vimentin and E-cadherin) in HFE lentiviral knockdown ARCaP$_M$ cells (KD$_{HFE1}$ and KD$_{HFE3}$). HFE knockdown ARCaP$_M$ cells underwent MET and had an epithelial-like phenotype.

lung, and renal cancer cells *in vitro* and *in vivo* and cause lethality in mice. We showed that (i) β2-M promoted EMT and its associated increase in cancer cell proliferation, migration, and invasion *in vitro*, and caused lethal skeletal and soft tissue metastases in mice; (ii) β2-M induced stable expression of EMT biomarkers, including decreased expression of E-cadherin and increased expression of N-cadherin and vimentin in cancer cells grown as primary and metastatic tumors in experimental mouse models; and (iii) β2-M forms a complex with its receptor HFE, which regulates intracellular iron and activates HIF-1α in cancer cells. To our knowledge, this is the first report to demonstrate how β2-M functionally confers increased cancer bone and soft tissue metastases in human prostate, breast, lung, and renal cancer cells by its induction of EMT in these cancer cells.

β2-M is a known growth-promoting protein for prostate (7, 10) and multiple myeloma (11) cells as well as normal bone cells, osteoblasts (17), osteoclasts (18), prostate stromal cells (10), and mesenchymal stem cells (16). β2-M was shown to promote osteomimicry in prostate cancer cells, allowing them to grow and survive in hostile bone microenvironments (7). Therefore it is not surprising that β2-M–overexpressing clones of prostate, breast, lung, and renal cancers had significantly increased bone metastases (Table 1) and lethality in experimental animals (Fig. 3B). β2-M may favor bone metastasis because firstly, increased β2-M expression in cancer cells promotes increased expression of bone matrix proteins such as osteocalcin and bone sialoprotein, mimicking the bone "niche" and supporting the growth and survival of prostate cancer cells in the bone microenvironment (7). Secondly, increased serum β2-M has been associated with increased bone remodeling which could trigger the secretion of soluble and matrix factors feeding further growth of cancer cells in the skeleton. Thirdly, β2-M could also promote the growth of osteoclasts (Fig. 2D), osteoblasts (31), and migrating mesenchymal stem cells (16) in the tumor microenvironment, further enhancing the growth of primary and metastatic cancer cells (32). Fourthly, β2-M could contribute to iron homeostasis and induction of HIF-1α in cancer cells (Fig. 6B and C) to promote the growth of cancer in the skeleton. Finally, β2-M has been proposed as a coupling factor between osteoclasts and osteoblasts (33) with a role in augmenting tumor and marrow stroma interaction, which could further activate a vicious cycle of metastatic cancer progression in bone (34).

β2-M mediates several hallmarks of malignancy, such as self-renewal capabilities, by activating phosphorylated cAMP response element binding protein, cyclin D1, and cyclin A (7), evading apoptosis by recruiting survival and growth factors and their receptors for downstream signaling (35), enhancing angiogenesis by activating VEGF-neuropilin signaling (7, 36), and inducing resistance to treatment and increasing stemness by activation of the HIF-1α signaling pathway (37). HIF-1α overexpression in tumor specimens is correlated with patient mortality (38). β2-M is upstream of HIF-1α, and induces a hypoxia-like effect through the reduction of iron levels. Here, we demonstrated that β2-M induces EMT and stemness-like properties in cancer cells.

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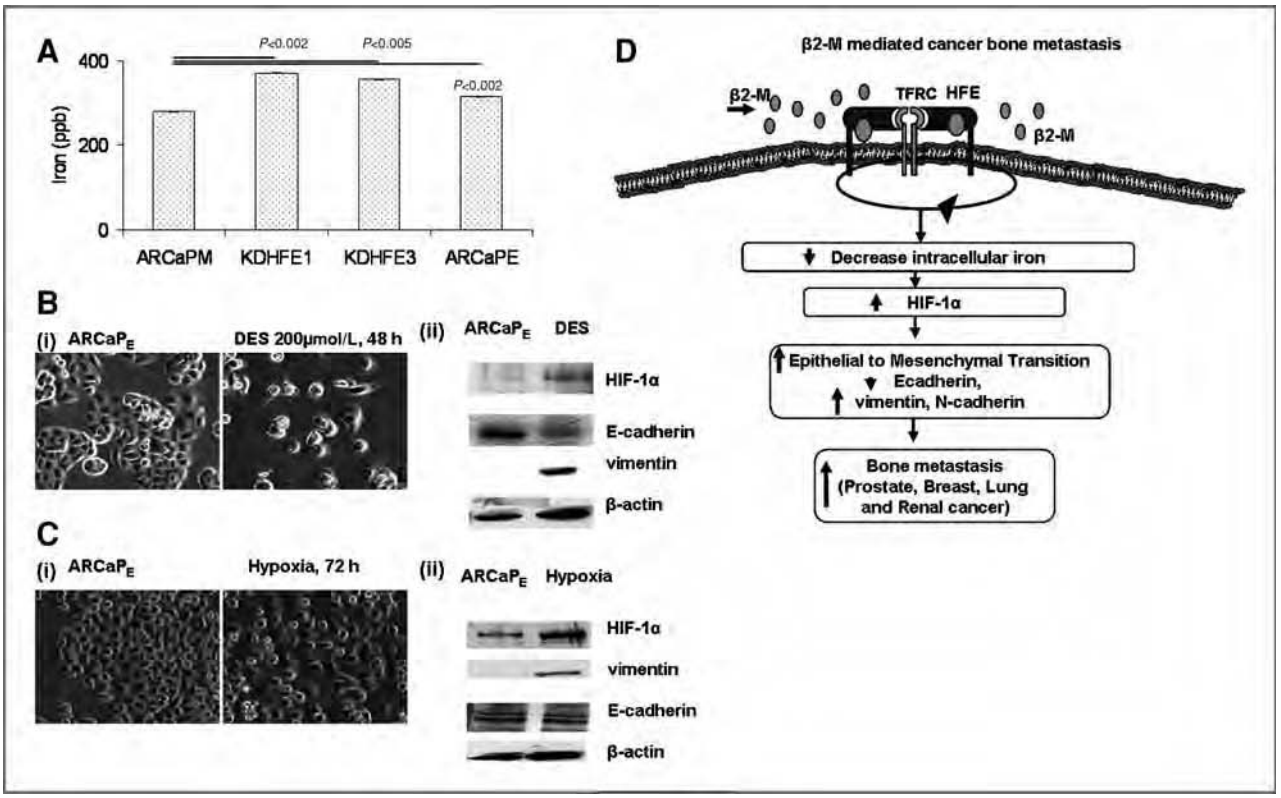

Figure 6. β2-M/HFE modulate iron levels to induce EMT. A, iron levels in $ARCaP_E$ and $ARCaP_M$ cells and HFE knockdown cells (KD_{HFE1} and KD_{HFE3}) measured by inductively coupled plasma mass spectroscopy. B, EMT changes in $ARCaP_E$ cells in response to iron chelation: (i) morphological changes and (ii) expression changes in EMT biomarkers. C, EMT changes in $ARCaP_E$ cells in response to hypoxia: (i) morphological changes and (ii) expression changes in EMT biomarkers. D, model of β2-M mediated bone metastasis. The β2-M/HFE complex modulated iron uptake by negative regulation of transferrin receptor 1. The β2-M/HFE complex maintains low iron levels and activates the EMT program via iron responsive pathways such as HIF-1α signaling. β2-M induces EMT and allows increased bone metastasis in prostate, breast, lung, and renal cancers.

In contrast to multiple myeloma, which expresses normal levels of MHC class 1 family members, β2-M interacts with MHC class 1 and mediates its downstream signaling processes by sequestering growth and survival signaling components mediated by lipid membrane and lipid rafts (11). In solid tumors, however, MHC class 1a members involved in antigen presentation are frequently downregulated. Thus MHC class 1b members, known to be involved in nonimmunological activities, are likely to mediate the β2-M downstream signaling functions of these tumor cells. HFE, a MHC class 1b protein shown to have a smaller groove and unable to present antigens (39), is likely to assume the signaling role of β2-M. β2-M/HFE has been shown to regulate negatively intracellular iron, activate HIF-1α and drive EMT in cancer cells. Our studies demonstrated that HFE is a β2-M receptor, since: (1) HFE was found to physically interact with β2-M, demonstrated by immunoprecipitation in prostate cancer cells (Fig. 5C) and (2) knocking down either HFE or β2-M resulted in MET, a reversal of EMT, in prostate cancer cells with supportive morphologic, biochemical, and behavioral characteristics. Thus β2-M/HFE interactions are important for β2-M mediated EMT and cell survival. The downstream functional significance of the β2-M/HFE complex is depicted in Figure 6D. β2-M/HFE plays a key role in regulating iron homeostasis in cancer cells, mediated by interacting with TFRC (transferrin receptor complex 1). β2-M protected the influx and accumulation of intracellular iron. Higher β2-M/HFE levels downregulate intracellular iron levels in $ARCaP_M$ cells and low levels of β2-M/HFE complex in $ARCaP_E$ cells enhanced intracellular iron levels (Fig. 6A). Lower levels of intracellular iron activated HIF-1α and its target genes in $ARCaP_M$ cells, driving EMT (30), which could contribute to resistance to treatments such as radiation and chemotherapy, resistance to apoptosis and increased angiogenesis (40). HIF-1α modulates the cell's redox balance by generating large levels of redox buffers such as glutathione and thioredoxin and alternatively activating NADPH oxidase enzymes as ROS generator and signaling molecules (37).

In summary, we demonstrated the importance of β2-M for cancer cell growth, invasion and metastasis. The action of β2-M is mediated by forming a complex with HFE which regulates intracellular iron homeostasis and HIF-1α and ultimately cancer metastasis to bone and soft tissues. The cell signaling network mediated by β2-M/HFE complex is highly conserved among several cancer cell types and deregulation of this

Published OnlineFirst March 22, 2011; DOI:10.1158/0008-5472.CAN-10-3382

complex could affect cancer growth and lethality in mice by the induction of EMT.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgment

The authors thank Mr. Gary Mawyer for his editorial assistance.

Grant Support

This study was supported by grants from P01-CA98912, DAMD-17-03-02-0033, and RO1-CA122602 to L.W.K. Chung.

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Received September 14, 2010; revised December 13, 2010; accepted January 5, 2011; published OnlineFirst March 22, 2011.

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